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(54) Title: METHODS FOR THE DETECTION OF CLONAL POPULATIONS OF TRANSFORMED CELLS IN A GENOMICALLY HETEROGENEOUS CELLULAR SAMPLE			
(57) Abstract Methods are provided for detecting the presence of mutant sequences in a subpopulation of gene sequences in a biological sample. These methods are particularly useful for identifying individuals with gene mutations indicative of early colorectal cancer.			

METHODS FOR THE DETECTION OF CLONAL POPULATIONS OF TRANSFORMED CELLS IN A GENOMICALLY HETEROGENEOUS CELLULAR SAMPLE

FIELD OF THE INVENTION

This invention relates to methods useful for disease diagnosis by detecting the presence of genetic mutations, including deletions, in cellular samples containing a small amount of mutated genetic material dispersed within a major amount of
5 diagnostically-irrelevant (normal) genetic material. Methods of the invention are especially useful in the detection of genetic mutations characteristic of cancer.

BACKGROUND OF THE INVENTION

Cancer is a disease characterized by genomic instability. Generally, genomic instability defines a broad class of disruptions in genomic nucleotide sequences. Such
10 disruptions include the loss of heterozygosity (usually characterized by massive loss of chromosomal DNA), microsatellite instability (usually indicative of defects in DNA repair mechanisms), and mutations (which include insertions, deletions, substitutions, duplications, rearrangements, or modifications). Numerous genomic instabilities have been associated with cancer. For example, mutations in a number of oncogenes and
15 tumor suppressor genes have been implicated in tumorigenesis. Duffy, *Clin. Chem.*, 41: 1410-1413 (1993). In addition, the loss of heterozygosity at the P53 tumor suppressor locus has been correlated with various types of cancer. Ridanpaa, *et al.*, *Path. Res. Pract.*, 191: 399-402 (1995). The loss or other mutation of the apc and dcc tumor suppressor genes has also been associated with tumor development. Blum,
20 *Europ. J. Cancer*, 31A: 1369-372 (1995). Finally, tumorigenesis has also been correlated with microsatellite instability.

Genetic changes characteristic of genomic instability theoretically can serve as markers for the early stages of, for example, colon cancer, and can be detected in DNA isolated from biopsied colonic epithelium and in some cases from transformed cells
25 shed into fecal material. Sidransky, *et al.*, *Science*, 256: 102-105 (1992).

Detection methods proposed in the art are time-consuming and expensive. Duffy, *supra*. Moreover, methods according to the art cannot be used to identify a loss of heterozygosity or microsatellite instability in small subpopulation of cells when the cells exist in a heterogeneous (i.e., clonally impure) sample. For example, in U.S. Patent No. 5,527,676, it is stated that tissue samples in which a mutation is to be detected should be enriched for tumor cells in order to detect the loss of heterozygosity in a p53 gene.

Techniques, such as PCR, have been used to detect a loss of heterozygosity resulting from massive deletions characteristic of late-stage adenomas. See, e.g., U.S. Patent No. 5,330,892. Such techniques generally require the use of large numbers of primer pairs, and they will not work at all in a heterogeneous sample. A recent publication reports the use of PCR and ELISA techniques to perform quantitative analysis of mutations in early-stage tumors. U.S. Patent No. 5,512,441. Identification of an abnormal subpopulation of cells in a heterogeneous sample of mostly normal cells and cellular debris, which subpopulation is characterized by loss of heterozygosity or microsatellite instability, is even more difficult because such detection involves the identification of a subpopulation of nucleotide fragments that are difficult to distinguish from the sea of heterogeneous normal cellular material in which they exist. A further problem is that mutation at any locus of a growing number of different oncogenes or tumor suppressor genes can result in cancer, and a screening approach capable of scanning all or even most loci in these genes is not currently available.

Microsatellite instability may also be a marker for cancer. Microsatellites are dispersed throughout the genome at an average frequency of about 1 in 100,000 base pairs. They comprise tandem or trinucleotide repeats that normally are inherited in a stable fashion. See, e.g., Charlesworth, *et al.*, *Nature*, 371: 215-220 (1994). While these sequences perform no known function in the genome, many of them have been mapped and have been used as markers based upon their sequence length polymorphisms. Clonal changes in microsatellite DNA associated with defects in mismatch repair pathways are thought to be suitable markers for hereditary non-polyposis colorectal cancer (HNPCC). In HNPCC tumor samples, for example, Microsatellites were found to have multiple insertions and/or deletions. Microsatellite instability may be an effective marker for failure of mismatch repair in oncogenes or in

tumor suppressor genes. While microsatellite instability itself is not indicative of cancer, it is evidence that mutations may occur in regions that are critical for onset of cancer. It follows that detection of instability in Microsatellites indicates that the patient is at risk of developing a clonal subpopulation of cancer cells.

5 Colorectal cancer is a common cause of death in Western society. Any tumor or precancerous polyp that develops along the length of the colon or the rectum sheds cells or DNA from cells into the lumen of the colon. Shed cells or cellular DNA are usually incorporated on stool as stool passes through the colon. In the early stages of cancer, cancerous or precancerous cells represent a very small fraction of the shed
10 epithelial cells or DNA in stool. Current methods for detection of colorectal cancer do not focus on detecting cancerous or precancerous cells in stool. Rather, such methods typically focus on extracellular indicia of the presence of cancer, such as the presence of fecal occult blood or carcinoembryonic antigen circulating in serum.

 It is known, however, that both sporadic and hereditary colorectal cancers result
15 from mutations in oncogenes and tumor suppressor genes. Such mutations appear to occur at a point in the etiology of the disease that is much earlier than the point at which extracellular indicia or clinical signs of cancer are observed. If detected early, colon cancer may be effectively treated by surgical removal of the cancerous tissue. Surgical removal of early-stage colon cancer is usually successful because colon
20 cancer begins in cells of the colonic epithelium and is isolated from the general circulation until the occurrence of invasion through the epithelial lining. Thus, detection of early mutations in colorectal cells would greatly increase survival rate.

 Current non-invasive methods for detection of colon cancer involve the detection of fecal occult blood and carcinoembryonic antigen. These screening methods often
25 either fail to detect colorectal cancer or they detect colorectal cancer only after it has progressed to an untreatable stage. Moreover, carcinoembryonic antigen is thought not to be an effective predictor of cancer but merely an indicator of recurrent cancer.

 Invasive techniques, such as endoscopy, while effective, are expensive and painful and suffer from low patient compliance. Accordingly, current colon cancer
30 screening methods are not practical for screening large segments of the population. See, e.g., Blum, *Europ. J. Cancer*, 31A: 1369-1372 (1995).

Therefore, there is a need in the art for simple and efficient non-invasive methods for reliable large-scale screening to identify individuals with early stage colon cancer. Such methods are provided herein.

SUMMARY OF THE INVENTION

5 The present invention provides methods for detecting a subpopulation of genomically transformed cells or cellular debris. Such methods detect the presence in a biological sample of a clonal subpopulation of cells which have a genome different from that of the wild type, and from bacterial, parasitic, or contaminating organisms that may also be present in the sample. Practice of the invention permits, for example,
10 detection of a trace amount of DNA derived from cancer or precancer cells in a biological sample containing a majority of "normal" DNA or whole cells. A preferred use of the methods is to reliably detect in a stool sample voided by a patient the presence of a trace amount of cells and/or cellular debris containing DNA shed into the colon at the site of an asymptomatic precancerous or cancerous lesion. The invention takes
15 advantage of several important insights which permit, for example, reliable detection of a DNA *deletion* at a known genomic site characteristic of a known cancer cell type.

In general, the invention comprises the comparative measurement of two genomic sequences. One genomic sequence is stable through transformation, (i.e., it is identical in both malignant and wild type cells in the sample). A second genomic
20 sequence typically undergoes change during the course of transformation (i.e., it is mutated during the development of malignant precursor cells). Hybridization probes are used to detect the presence of each genomic sequence. If the number of hybridization events involving the two genomic sequences is different, the difference may be due to insignificant background or it may be due to a statistically-significant
25 difference in the quantities of the two genomic sequences in the population from which the sample was drawn. In the latter case, the difference can be correlated, to a degree of defined statistical confidence, with the presence in the sample of a subpopulation of cells having an altered (i.e., non-wild type) genomic sequence.

The invention may be divided into three general embodiments. (1) In a first
30 general embodiment, a quantitative amount (number of copies) of a gene or gene fragment of interest in a sample (i.e. a gene the mutation of which is known or

suspected to be associated with cancer) is compared to a quantitative amount of a reference gene or gen fragment in the sample, the reference gene being a gene which is not normally associated with cancer and which normally has a low rate of mutation. A statistically-significant difference between the two quantitative amounts is indicative of genomic instability in a cellular subpopulation in the sample. (2) In a second general embodiment of the invention, a quantitative amount of a region on a maternal allele is compared to a quantitative amount of the corresponding region on a paternal allele. A statistically-significant difference between the two quantitative amounts is indicative of genomic instability. (3) In a third general embodiment, the number of microsatellite repeats at a particular locus are compared between maternal and paternal alleles. A statistically-significant difference in those numbers is indicative of an error in the mismatch repair mechanisms in a subpopulation of cells in the sample or may indicate that allelic loss has taken place. As stated above, errors in mismatch repair may result in mutations in tumor suppressor genes or in oncogenes. Cancer detection in any of the three embodiments of the invention is achieved by measuring the *number* of hybridizations between at least two different nucleotide probes and their respective genomic sequences.

One feature of the invention is that it has now been recognized that materials from cells lining the colon (e.g., a polyp or lesion) are shed onto forming stool only in a region comprising a longitudinal stripe along the length of the stool. Thus, unless the stool sample under investigation is a whole stool or comprises at least a cross-section of a stool, the sample will contain the relevant diagnostic information only by chance. The colon contains numerous bends and folds throughout its length. See, U.S. Patent Number 5,741,650. Epithelial cells lining the colon normally migrate from a basal position in colonic crypts, where stem cells divide by mitosis, to the top of the crypts and are then shed into the lumen. Colonic epithelial cells that line the intestinal lumen typically undergo regeneration every four to five days as a result of the rapid turnover rate through the epithelium. Accordingly, sloughed epithelial cells or their DNA are constantly being deposited in the forming stool as it passes through the lumen. As the stool proceeds toward the rectum and becomes progressively more solid (from an initial liquid state), epithelial cells are only sloughed onto the portion of the stool making



contact with the portion of the lumen that formerly contained those cells in its epithelial lining. Epithelial cells of a polyp (a polyp is a pre-cancerous growth; while not all polyps become cancerous, almost all cancers arise from polyps) undergo the same rapid life cycle and shedding described above for normal colonic epithelial cells.

- 5 Accordingly, cells shed from polyps are typically only absorbed onto the surface of the forming stool that makes contact with the polyp. However, if the stool is in a liquid state, mixing of shed polyp cells throughout the stool occurs automatically.

Accordingly, the present invention provides methods for detecting genomic changes in a subpopulation of cells in a sample of biological material. Methods of the invention are useful for the detection of changes in the nucleotide sequence of an allele in a small subpopulation of cells present in a large, heterogeneous sample of diagnostically-irrelevant biological material. Methods of the invention are useful for the detection and diagnosis of a genetic abnormality, such as a loss of heterozygosity or, more generally, a mutation, which can be correlated with a disease, such as cancer.

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15 For purposes of the present invention, unless the context requires otherwise, a "mutation" includes modifications, rearrangements, deletions, substitutions, and additions in a portion of genomic DNA or its corresponding mRNA.

In a preferred embodiment, the invention provides methods for detecting a clonal subpopulation of transformed cells contained in, or suspected of being contained in, a biological sample obtained from an organism, such as a human. The methods comprise the steps of determining from the biological sample a number X of a first wild-type polynucleotide that is known or suspected not to be mutated in either wild-type cells or in transformed cells. A further step comprises determining from the biological sample a number Y of a second wild-type polynucleotide suspected of being mutated in a subpopulation of cells in the biological sample. Then, one determines whether a statistically-significant difference exists between X and Y. In a normal sample there is no mutation and so there is no statistically-significant difference between the number of each of the somatic genes in a normal cell. As a result, X and Y are not different from each other in a statistically significant sense. In contrast, the presence of a statistically significant difference between X and Y is indicative of the presence of a clonal subpopulation of transformed cells in the biological sample embodying a mutation. Statistical significance may be determined by any method known in the art.

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However, no formal measurement of statistical significance need be performed in connection with any given assay. Rather, assays are designed to detect a large enough number of binding events such that at least a threshold difference between the numbers is dispositive of the issue of the presence of a mutant subpopulation of cells at any desired level of certainty.

Also, in a preferred embodiment, transformed cells sought to be detected using methods according to the invention are malignant cells. Transformed cells detected according to methods of the invention may be induced transformants, transformed, for example, by a virus, by radiation, or by chemical or other carcinogenic means.

Methods of the invention may be performed on any biological sample, including tissue and body fluid samples. Particularly preferred biological samples include pus, sputum, semen, blood, saliva, cerebrospinal fluid, and urine. In an important embodiment of the invention the sample is stool which is analyzed to detect colorectal cancer or precancer. Methods of the invention may be practiced by exposing the biological sample to one or more oligonucleotide probes in order to separately detect the number X of a first polynucleotide and the number Y of a second polynucleotide. Probes for use in the invention are detectably labeled. Preferred labels include fluorescent labels attached, for example, by affinity binding pairs (such as carbohydrate/lectin or avidin/biotin). Highly-preferred labels are microscopic particles which are counted by a detection apparatus, preferably a high-speed electronic apparatus as disclosed herein. The numbers X and Y are preferably proportional and most preferably equal to the number of target polynucleotide detection events occurring in the biological sample.

Methods of the invention are especially useful for the detection of colorectal cancer or precancerous cells in humans. For purposes of the present invention, precancerous cells are cells that have a mutation that is associated with cancer, and which renders such cells susceptible to becoming cancerous. Such methods comprise determining whether cells or nucleotide debris in a stool sample include a deletion of a polynucleotide normally present in a wild-type genome of the human or other mammal. The sample may be exposed to a plurality of first and second oligonucleotide probes under hybridization conditions, thereby to hybridize (i) first probe to copies of a first polynucleotide segment characteristic of a wild-type genomic region known or suspected not to be deleted in cells of the sample and (ii) second probe to copies of a

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second polynucleotide segment characteristic of the wild-type genomic region suspected of being mutated in the sample. The number of duplexes formed with each of the first and second probes is then detected and counted. The presence of a statistically-significant difference in those two numbers is indicative of the presence in the sample of a mutation that may be characteristic of colorectal cancer. Endoscopy or other visual examination procedures are then indicated.

In a preferred embodiment, probes are labeled with beads or particles. In this embodiment, probes used for detection of genomic polynucleotide segments in a sample are preferably bound to such beads in a ratio of one probe to one bead, and the beads linked to the first and second probes are distinguishable, for example, by size. Use of such hybridization beads or particles facilitates the quantitative detection of genomic polynucleotide segments in the sample using, for example, an impedance counter, such as a "Coulter counter".

Methods according to the invention also may be used to detect a loss of heterozygosity at an allele by determination of the amounts of maternal and paternal alleles comprising a genetic locus that includes at least one single-base polymorphism. A statistically-significant difference in the amounts of each allele is indicative of a mutation in an allelic region encompassing the single-base polymorphism. In this method, a region of an allele comprising a single-base polymorphism is identified, using, for example, a database, such as GenBank, or by other means known in the art. Probes are designed to hybridize to corresponding regions on both paternal and maternal alleles immediately 3' to the single base polymorphism as shown in Figure 3. After hybridization, a mixture of at least two of the four common dideoxy nucleotides are added to the sample, each labeled with a different detectable label. A DNA polymerase is also added. Using allelic DNA adjacent the polymorphic nucleotide as a template, hybridized probe is extended by the addition of a single dideoxynucleotide that is the binding partner for the polymorphic nucleotide. After washing to remove unincorporated dideoxynucleotides, the dideoxynucleotides which have been incorporated into the probe extension are detected by determining the number of bound extended probes bearing each of the two dideoxy nucleotides in, for example, a flow cytometer or impedance counter. The presence of an almost equal number of two different labels mean that there is normal heterozygosity at the polymorphic nucleotide.

The presence of a statistically-significant difference between the detected numbers of the two labels means that a deletion of the region encompassing the polymorphic nucleotide has occurred in one of the alleles.

Methods of the invention may be used to determine whether a patient is a
5 candidate for follow-up invasive diagnostic or other procedures, such as endoscopy. For example, methods of the invention may be used to detect a mutation in a tumor suppressor gene or an oncogene in a subpopulation of cells in a stool sample obtained from a patient. An endoscopy procedure may then be performed on patients diagnosed with a mutation. A positive endoscopy result is then followed by polypectomy, surgery,
10 or other treatment to remove cancerous or precancerous tissue.

Accordingly, it is an object of the invention to provide methods for detecting genomic instability in a subpopulation of cells in a cellular sample. It is a further object of the invention to provide methods for detecting a genomic change in a subpopulation of cells, wherein the genomic change is indicative of cancer. It is another object of the
15 invention to detect a loss of heterozygosity in a genomic region associated with cancer, such as a tumor suppressor region. It is yet another object of the invention to provide methods for detecting heterozygosity and the loss thereof at single-base polymorphic nucleic acids. Finally, it is an object of the invention to provide methods for the detection of cancer, and particularly colorectal cancer by detection of cells or cellular
20 debris indicative of cancer in a heterogeneous sample, such as a stool sample.

Further aspects of the invention will become apparent upon consideration of the following detailed description and of the drawings.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart showing sequential steps in methods of the invention.

25 Figure 2 is a schematic diagram of a multi-orifice impedance counter of the type useful in accordance with the invention to count hybridization events; wherein reference numeral 1 indicates the direction of flow through the column; reference numeral 2 indicates a plunger means for forcing material downward in the column; reference numerals 3 and 4 are different-sized hybridization beads; reference numeral
30 5 is an optional filter for extracting unwanted particles; reference numeral 6 indicates

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an array of orifices for measuring differential impedance; and reference numeral 7 is a collection chamber.

Figure 3 shows four possible probe attachment sites on allelic regions characterized by having a single base polymorphism. In Figure 3, sequence M1 is
5 SEQ ID NO:1; sequence M2 is SEQ ID NO: 2; sequence M3 is SEQ ID NO:3; sequence M4 is SEQ ID NO: 4; sequence F1 is SEQ ID NO: 5; sequence F2 is SEQ ID NO: 6; sequence F3 is SEQ ID NO: 7; sequence F4 is SEQ ID NO: 8.

Figures 4A and 4B are model Gaussian distributions showing regions of low statistical probability.

10 Figure 5 is graph showing the probable values of N for a heterogeneous population of cells in which 1% of the cells are mutated.

DETAILED DESCRIPTION OF THE INVENTION

Methods according to the present invention are useful for the detection of genomic instability in a heterogeneous cellular sample in which the genomic instability
15 occurs in only a small subpopulation of cells in the sample. Using traditional detection methods, such a subpopulation would be difficult, if not impossible, to detect - especially if the mutation that is causative of genomic instability is unknown at the time of detection or a clonally-impure cellular population is used. See, e.g., U.S. Patent No. 5,527,676 (reporting that a clonal population of cells should be used in order to
20 detect a deletion in a p53 gene). Traditional methods for detection of mutations involved in carcinogenesis rely upon the use of a clonally-pure population of cells and such methods are best at detecting mutations that occur at known "hot spots" in oncogenes, such as k-ras. See, Sidransky, supra. Using the PCR-based methods of the art, an extremely large number of primers would have to be designed and sample
25 would have to be tested numerous times to detect genomic instability in a cellular sample that is clonally-impure (i.e., a heterogeneous sample such as stool) and in which the mutation to be detected is unknown and exists in a very small number of cells. Moreover, PCR is not useful for detection of the absence of a genetic sequence, as in the case of the present methods for detecting loss of heterozygosity. Even after
30 such repeated testing, a PCR-based method may not detect a mutation in a small number of cells in a clonally-impure population if, for example, primers bordering the

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site of the mutation are not used. Thus, in early-stage adenomas (when the population of mutated cells is very small), methods of the art are, at best, impractical and may not work at all.

In contrast, methods of the present invention are capable of detecting genomic
5 instability in a small number of cells in an impure cellular population because such methods do not rely upon knowing which mutation exists and such methods are not affected by the presence in the sample of heterogeneous DNA. For example, in loss of heterozygosity, deletions occur over large portions of the genomes and entire alleles may be missing (or at least enough of an allele may be missing in order to render the
10 allele non-functional). Methods of the invention comprise counting a number of a gene suspected of being mutated and comparing that number with the number of a gene known not to be mutated in the same sample. All that one needs to know is at least a portion of the sequence of a wild-type gene in which the mutation is suspected to occur and at least a portion of the wild-type sequence of a reference gene in which mutation
15 is not suspected to have occurred.

Accordingly, methods of the present invention are useful for the detection of changes in a genomic nucleotide sequence present in a subpopulation of cells or debris therefrom in a sample. Such changes generally occur as a mutation (i.e., a substitution, modification, deletion, addition, or rearrangement) in a wild-type allelic
20 sequence in a subpopulation of cells. In the case of a tumor suppressor gene, the mutation typically takes the form of a massive deletion characteristic of loss of heterozygosity. Often, as in the case of certain forms of cancer, disease-causing mutations initially occur in a single cell which then produces a small subpopulation of mutant cells. By the time clinical manifestations of the mutation are detected, the
25 disease may have progressed to an incurable stage. Methods of the invention allow detection of a mutation when the mutation exists as only a small percentage of the total cells or cellular debris in a sample.

Methods of the invention comprise a comparison of two wild-type sequences that are expected to be present in the sample in equal numbers in normal (non-mutated)
30 cells. In a preferred embodiment, the comparison is between (1) an amount of a genomic polynucleotide segment that is known or suspected not to be mutated in cells of the sample (the "reference") and (2) an amount of a wild-type (non-mutated) genomic

polynucleotide segment suspected of being mutated in a subpopulation of cells in the sample (the "target"). A statistically-significant difference between the amounts of the two genomic polynucleotide segments indicates that a mutation has occurred.

Specifically, in the case of a deletion in a tumor suppressor gene, the detected amount
5 of the reference gene is significantly greater than the detected amount of the target gene. If a target sequence is amplified, as in the case of certain oncogene mutations, the detected amount of target is greater than the detected amount of the reference gene by a statistically-significant margin.

Methods according to the art generally require the use of numerous probes,
10 usually in the form of PCR primers and/or hybridization probes, in order to detect a deletion or a point mutation. However, because methods of the present invention involve quantitative detection of nucleotide sequences and quantitative comparisons between sequences that are known to be stable and those that are suspected of being unstable, only a few probes must be used in order to accurately assess cancer risk. In
15 fact, a single set (pair) of probes is all that is necessary. The risk of cancer is indicated by the presence of a mutation in a genetic region known or suspected to be involved in oncogenesis. Patients who are identified as being at risk based upon tests conducted according to methods of the invention are then directed to other, typically invasive, procedures for confirmation and/or treatment of the disease.

20 Quantitative sampling of a nucleotide sequence that is uniformly distributed in a biological sample typically follows a Poisson distribution. For large populations, such as the typical number of genomic polynucleotide segments in a biological sample, the Poisson distribution is similar to a normal (Gaussian) curve with a mean, N , and a standard deviation that may be approximated as the square root of N .

25 Statistically-significance between numbers of target and reference genes obtained from a biological sample may be determined by any appropriate method. See, e.g., Steel, *et al.*, Principles and Procedures of Statistics, A Biometrical Approach (McGraw-Hill, 1980), the disclosure of which is incorporated by reference herein. An exemplary method is to determine, based upon a desired level of specificity (tolerance
30 of false positives) and sensitivity (tolerance of false negatives) and within a selected level of confidence, the difference between numbers of target and reference genes that must be obtained in order to reach a chosen level of statistical significance. A

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threshold issue in such a determination is the minimum number, N, of genes (for each of target and reference) that must be available in a population in order to allow a determination of statistical significance. The number N will depend upon the assumption of a minimum number of mutant alleles in a sample containing mutant alleles (assumed herein to be at least 1%) and the further assumption that normal samples contain no mutant alleles. It is also assumed that a threshold differences between the numbers of reference and target genes must be at least 0.5% for a diagnosis that there is a mutation present in a subpopulation of cells in the sample. Based upon the foregoing assumptions, it is possible to determine how large N must be so that a detected difference between numbers of mutant and reference alleles of less than 0.5% is truly a negative (i.e. no mutant subpopulation in the sample) result 99.9% of the time.

The calculation of N for specificity, then, is based upon the probability of one sample measurement being in the portion of the Gaussian distribution covering the lowest 3.16% of the population (the area marked "A" in figure 4A) and the probability that the other sample measurement is in the portion of the Gaussian distribution covering the highest 3.16% of the population (the area marked "B" in figure 4B). Since the two sample measurements are independent events, the probability of both events occurring simultaneously is approximately 0.001 or 0.1%. Thus, 93.68% of the Gaussian distribution (100% - 2x3.16%) lies between the areas marked A and B in figure 5A. Statistical tables indicate that such area is equivalent to 3.72 standard deviations. Accordingly, 0.5%N equals 3.72 sigma. Since sigma (the standard deviation) is equal to \sqrt{N} , the equation may be solved for N as 553,536. This means that if the lower of the two numbers representing reference and target is at least 553,536 and if the patient is normal, the difference between the numbers will be less than 0.5% about 99.9% of the time.

To determine the minimum N required for 99% sensitivity a similar analysis is performed. This time, one-tailed Gaussian distribution tables show that 1.28 standard deviations (sigma) from the mean cover 90% of the Gaussian distribution. Moreover, there is a 10% (the square root of 1%) probability of one of the numbers (reference or target) being in either the area marked "A" in figure 5 or in the area marked "B" in figure

5. If the two population means are a total of 1% different and if there must be a 0.5% difference between the number of target and reference genes, then the distance from either mean to the threshold for statistical significance is equivalent to 0.25%N (See Figure 5) for 99% sensitivity. As shown in Figure 5, 0.25%N corresponds to about
5 40% of one side of the Gaussian distribution. One-tailed statistical tables reveal that 40% of the Gaussian distribution corresponds to 1.28 standard deviations. Therefore, 1.28 sigma is equal to 0.0025N, and N equals 262,144. Thus, for abnormal samples, the difference will exceed 0.5% at least 99% of the time if the lower of the two numbers is at least 262,144. Conversely, an erroneous negative diagnosis will be made only 1%
10 of the time under these conditions.

In order to have both 99.9% specificity (avoidance of false positives) and 99% sensitivity (avoidance of false negatives), a sample with at least 553,536 (or roughly greater than 550,000) of both target and reference alleles should be used. A difference of at least 0.5% between the numbers obtained is significant at a confidence level of
15 99.0% for sensitivity and a difference of less than 0.5% between the numbers is significant at a confidence level of 99.9% for specificity. As noted above, other standard statistical tests may be used in order to determine statistical significance and the foregoing represents one such test.

Based upon the foregoing explanation, the skilled artisan appreciates that
20 methods of the invention are useful to detect mutations in a subpopulation of a polynucleotides in any biological sample. For example, methods disclosed herein may be used to detect allelic loss (the loss of heterozygosity) associated with diseases such as cancer. Additionally, methods of the invention may be used to detect a deletion or a base substitution mutation causative of a metabolic error, such as complete or partial
25 loss of enzyme activity. For purposes of exemplification, the following provides details of the use of methods according to the present invention in colon cancer detection. Inventive methods are especially useful in the early detection of a mutation (and especially a large deletion typical of loss of heterozygosity) in a tumor suppressor gene. Accordingly, while exemplified in the following manner, the invention is not so
30 limited and the skilled artisan will appreciate its wide range of applicability upon consideration thereof.

Methods according to the invention preferably comprise one of three types of detection regimens. In a first preferred detection regimen, an amount of a polynucleotide known or suspected to be mutated is compared to an amount of a reference polynucleotide known or suspected not to be mutated. In a second
5 preferred detection regimen, an amount of a polymorphic nucleotide on a maternal allele is compared to an amount of the corresponding polymorphic nucleotide on the corresponding paternal allele. Finally, a third detection regimen comprises a comparison of a microsatellite repeat region in a normal allele with the corresponding microsatellite region in an allele known or suspected to be mutated. All three
10 exemplary detection means comprise determining whether a difference exists between the amounts of each nucleic acid being measured. The presence of a statistically-significant difference is indicative that a mutation has occurred in one of the nucleic acids being measured. Thus, methods described below are generally applicable to all forms of the invention, the variations of which are shown in figure 1.

15 **I. Preparation of a Stool Sample**

A sample prepared from stool voided by a patient should comprise at least a cross-section of the voided stool. As noted above, stool is not homogenous with respect to sloughed cells. As stool passes through the colon, it absorbs sloughed cells from regions of the colonic epithelium with which it makes contacts. Thus, sloughed
20 cells from a polyp are absorbed on only one surface of the forming stool (except near the cecum where stool is still liquid and is homogenized by Intestinal Peristalsis). Taking a representative sample of stool (i.e., at least a cross-section) and homogenizing it ensures that sloughed cells from all epithelial surfaces of the colon will be present for analysis in the processed stool sample. Stool is voided into a receptacle
25 that is preferably small enough to be transported to a testing facility. The receptacle may be fitted to a conventional toilet such that the receptacle accepts stool voided in a conventional manner. The receptacle may comprise a mesh or a screen of sufficient size and placement such that stool is retained while urine is allowed to pass through the mesh or screen and into the toilet. The receptacle may additionally comprise
30 means for homogenizing voided stool. Moreover, the receptacle may comprise means for introducing homogenization buffer or one or more preservatives, such as alcohol or

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a high salt concentration solution, in order to neutralize bacteria present in the stool sample and to inhibit degradation of DNA.

The receptacle, whether adapted to fit a toilet or simply adapted for receiving the voided stool sample, preferably has sealing means sufficient to contain the voided stool sample and any solution added thereto and to prevent the emanation of odors. The receptacle may have a support frame which is placed directly over a toilet bowl. The support frame has attached thereto an articulating cover which may be placed in a raised position, for depositing of sample or a closed position (not shown) for sealing voided stool within the receptacle. The support frame additionally has a central opening traversing from a top surface through to a bottom surface of the support frame. The bottom surface directly communicates with a top surface of the toilet. Extending from the bottom surface of the support frame and encompassing the entire circumference of the central opening is a means for capturing voided stool. The means for capturing voided stool may be fixedly attached to the support frame or may be removably attached for removal subsequent to deposition of stool.

Once obtained, the stool sample is homogenized in an appropriate buffer, such as phosphate buffered saline or a chaotropic salt solution. Homogenization means and materials for homogenization are generally known in the art. See, e.g., U.S. Patent No. 4,101,279. Thus, particular homogenization methods may be selected by the skilled artisan. Methods for further processing and analysis of a biological sample, such as a stool sample are presented below.

II. Methods for Detection of Colon Cancer or Precancer

A. Reference-Target

For exemplification, methods of the invention are used to detect a deletion or other mutation in the p53 tumor suppressor gene in cells obtained from a representative stool sample. The p53 gene is a good choice because the loss of heterozygosity in p53 is often associated with colorectal cancer. An mRNA sequence corresponding to the DNA coding region for p53 is reported as GenBank Accession No. M92424. The skilled artisan understands that methods described herein may be used to detect mutations in any gene and that detection of a p53 deletion is exemplary of such methods. At least a cross-section of a voided stool sample is obtained and

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prepared as described immediately above. DNA or RNA may optionally be isolated from the sample according to methods known in the art. See, Smith-Ravin, *et al.*, *Gut*, 36: 81-86 (1995), incorporated by reference herein. However, methods of the invention may be performed on unprocessed stool.

5 Nucleic acids may be sheared or cut into small fragments by, for example, restriction digestion. The size of nucleic acid fragments produced is not critical, subject to the limitations described below. A target allele that is suspected of being mutated (p53 in this example) and a reference allele are chosen. A reference allele may be any allele known or suspected not to be mutated in colon cancer cells. Single-stranded
10 nucleic acid fragments may be prepared using well-known methods. See, e.g., Sambrook, *et al.*, *Molecular Cloning, A Laboratory Manual* (1989) incorporated by reference herein.

Either portions of a coding strand or its complement may be detected in methods according to the invention. For exemplification, detection of the coding strand of p53
15 and reference allele are described. Complement to both p53 and reference allele are removed by hybridization to anti-complement oligonucleotide probes (isolation probes) and subsequent removal of duplex formed thereby. Methods for removal of complement strands from a mixture of single-stranded oligonucleotides are known in the art and include techniques such as affinity chromatography. Upon converting
20 double-stranded DNA to single-stranded DNA, sample is passed through an affinity column comprising bound isolation probe that is complementary to the sequence to be isolated away from the sample. Conventional column chromatography is appropriate for isolation of complement. An affinity column packed with sepharose or any other appropriate materials with attached complementary nucleotides may be used to isolate
25 complement DNA in the column, while allowing DNA to be analyzed to pass through the column. See Sambrook, *Supra*. As an alternative, isolation beads may be used to exclude complement as discussed in detail below.

After removal of complement strands, first oligonucleotide probes which hybridize to at least a portion of the p53 allele and second oligonucleotide probes that
30 hybridize to at least a portion of the reference allele are obtained. The probes are labeled with a detectable label, such as fluorescein or detectable particles. Distinct labels for the probes are preferred.

Labeled probes are then exposed to sample under hybridization conditions. Such conditions are well-known in the art. See, e.g., Wallace, et al., *Nucleic Acids Res.*, 6:3543-3557 (1979), incorporated by reference herein. First and Second oligonucleotide probes that are distinctly labeled (i.e. with different radioactive isotopes, fluorescent means, or with beads of different size, See *infra*) are applied to a single aliquot of sample. After exposure of the probes to sample under hybridization conditions, sample is washed to remove any unhybridized probe. Thereafter, hybridized probes are detected separately for p53 hybrids and reference allele hybrids. Standards may be used to establish background and to equilibrate results. Also, if differential fluorescent labels are used, the number of probes may be determined by counting differential fluorescent events in a sample that has been diluted sufficiently to enable detection of single fluorescent events in the sample. Duplicate samples may be analyzed in order to confirm the accuracy of results obtained.

If there is a statistically-significant difference between the amount of p53 detected and the amount of the reference allele detected, it may be assumed that a mutation has occurred in p53 and the patient is at risk for developing or has developed colon cancer. Statistical significance may be determined by any known method. A preferred method is outlined above.

The determination of a p53 mutation allows a clinician to recommend further treatment, such as endoscopy procedures, in order to further diagnose and, if necessary, treat the patient's condition. The following examples illustrate methods of the invention that allow direct quantification of hybridization events.

1. Method for Increased Quantitation of Target and Reference Polynucleotides

Enhanced quantification of binding events between hybridization probes and target or reference is accomplished by coupling hybridization probes to particles, such as beads (hybridization beads).

In order to obtain a precise quantitative measure of the amount of a polynucleotide in a sample, hybridization beads are constructed prior to conducting hybridizations, such that each bead has attached thereto a single oligonucleotide probe.

a. *Method for Preparation of Probe-Bead Combinations*

A single probe is attached to a bead by incubating a large excess of hybridization beads with oligonucleotide probes of a given type (*i.e.*, either first or second oligonucleotide probes). Coupling of probe to bead is accomplished using an affinity-binding pair. For example, beads may be coated with avidin or streptavidin and probes may be labeled with biotin to effect attachment of the probe to the bead. The mixture of beads and probes is agitated such that virtually 100% of the probes are bound to beads. The mixture is then exposed to a matrix, such as an affinity column or a membrane coated with oligonucleotides that are complementary to the probe. Only beads that have an attached probe will adhere to the matrix, the rest being washed away. Beads with coupled probe are then released from the matrix by melting hybridizations between probe and complement. Multiple exposures to the matrix and pre-washing of the column reduces non-specific binding. Moreover, naked beads (*i.e.*, without attached probe) may be exposed to the matrix to determine a background number of beads that can be expected to attach to the matrix in the absence of probe.

By using a vast excess of beads relative to probe as described above, it is expected that the vast majority of recovered beads will have only one attached probe. For example, if a mixture has a ratio of 1 probe to 1000 beads, it is expected that only about 1 bead in a million will have two attached probes and even less than one bead in a million will have more than two attached probes. Accordingly, hybridization beads are provided in an effective 1:1 ratio with probe which allows for precise quantification of target and reference polynucleotide as described below.

For each assay described below, two distinct hybridization beads are used. A first hybridization bead has attached thereto a single first oligonucleotide probe that is complementary to at least a portion of a target polynucleotide (*e.g.*, a p53 allele). A second hybridization bead, of a size distinct from the first hybridization bead, has attached thereto a single second oligonucleotide probe that is complementary to at least a portion of a reference polynucleotide (*i.e.*, one that is known or suspected not to be mutated in the sample).

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b. *Use of Beads to Quantify Target and Reference Polynucleotides*

DNA is melted (denatured to form single-stranded DNA) by well-known methods See, e.g., Gyllenstein, et al., in *Recombinant DNA Methodology II*, 565-578 (Wu, ed., 5 1995), incorporated by reference herein. According to methods of the invention, one may detect either a coding strand or its complement in order to quantify target and/or reference polynucleotide. For purposes of illustration, the present example assumes detection of the coding strand.

2. *Removal of Complement*

10 Single-stranded complement of the target polynucleotide (e.g., p53) and reference polynucleotide are removed from the sample by binding to oligonucleotide probes that are complementary to target or reference complement. Such probes, referred to herein as isolation probes, are attached to isolation beads prior to their introduction into the sample. The beads may be magnetized. Thus, when magnetized

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